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(54) Title: A METHOD FOR MAINTAINING EMBRYONIC STEM CELLS AND AVIAN FACTOR USEFUL FOR SAME

(57) Abstract

The present invention provides a method for maintaining *in vitro* animal embryonic stem (ES) cells without substantial differentiation, said method comprising culturing said ES cells in the presence of a feeder layer comprising chicken embryonic fibroblasts. The present invention also contemplates an isolated chick-derived ES stem cell factor obtainable from chicken embryonic fibroblasts and chick embryonic extracts.

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A METHOD FOR MAINTAINING EMBRYONIC STEM CELLS AND AVIAN FACTOR USEFUL FOR SAME

The present invention is directed generally to an avian factor and more particularly an avian factor useful in supporting propagation and/or maintenance of animal embryonic stem cells.

The development of methods to produce transgenic animals are an important means of gaining a better understanding of genome organisation and also for improving or introducing desirable traits in commercial animal production. A number of approaches have been proposed to facilitate gene transfer based on the observation that totipotent cells in the early embryo are susceptible to manipulation and introduction of foreign DNA. Such approaches have included gene transfer via retroviral vectors, sperm-mediated transfer, PGC transfer and microinjection.

In work leading up to the present invention, the inventors investigated the establishment of embryonic stem (ES) cells as a means of producing transgenic animals. ES cells offer advantages over other methods of producing transgenic animals since they are capable of *in vitro* genetic manipulation such as targeted mutagenesis by selective inactivation or replacement of endogenous genes and/or the introduction of genes or genetic sequence encoding new traits.

However, on culture, embryonic stem cells undergo differentiation and lose their stem cell phenotype. Previously this has been overcome by culturing ES cells on a feeder layer of fibroblasts (1).

Primary embryonic mouse fibroblasts and immortalised mouse STO cells have been used as feeder cells in the isolation and maintenance of murine ES cells (2). It has been reported that STO cells have also been used to successfully isolate and maintain porcine ES cells (3). Leukaemia Inhibitory Factor (LIF) has been implicated in the maintenance of ES cells in culture and has subsequently been isolated from mouse (4, 5, 6, 7) and human (8) sources.

Purified recombinant LIF (rLIF) is also routinely used to maintain mouse ES cells in the absence of feeder cells or conditioned medium (5).

ES cells cultured for a number of generations in the presence of mouse feeder cells or rLIF have a reduced capacity to contribute to the germ line (loss of totipotency) when these cultured cells are introduced into an early embryo. There is accordingly a need for factors capable of maintaining ES cells in culture without substantial differentiation.

Accordingly, one aspect of the present invention is directed to a method for maintaining *in vitro* animal embryonic stem (ES) cells without substantial differentiation said method comprising culturing said ES cells in the presence of a feeder layer comprising chicken embryonic fibroblasts.

More preferably, the present invention contemplates a method for maintaining *in vitro* embryonic stem (ES) cells from mice, rats, chickens, pigs, sheep, cattle, birds, and other non-human animals without substantial differentiation said method comprising culturing said ES cells in the presence of a feeder layer comprising chicken embryonic fibroblasts.

In a related aspect of the present invention, there is provided a method for maintaining *in vitro* animal ES cells without substantial differentiation comprising culturing said ES cells under appropriate conditions and in the presence of an effective amount of an avian factor. In a preferred aspect of the present invention, the avian factor is derived from chicken embryonic fibroblasts (CEF) or other embryonic tissue such as chick embryo extract (CEE). In a most preferred aspect of the present invention, the avian factor is a cytokine or cytokine-like molecule.

The present invention is predicated, at least in part, on the discovery that CEF and CEE produce a factor which maintain ES cells in the substantially undifferentiated state. The present invention extends to the maintenance of all animal ES cells including cells from

mammals such as humans, livestock animals, companion animals, laboratory test animals and wild animals as well as all avian ES cells including cells from poultry, game birds, caged birds and wild birds. The use of non-chicken ES cells is dependent on CEF or the factor produced therein or in CEE having activity in maintaining the ES cells which could be readily tested by one skilled in the art. In a preferred embodiment, the ES cells are of mouse, rat, chicken, pig, sheep or cattle origin.

The invention further extends to the maintenance in culture of human or animal primordial germ cells, haemopoietic stem cells, or cell lineage stem cells, without substantial differentiation utilising CEF or avian factor as herein described, optionally in association with other cytokine factors, such as steel factor (21).

The CEF of the present invention are a primary cell line with a finite life in culture. They form a confluent monolayer of fibroblastoid cells. The CEF may be prepared from "naturally" occurring cells or the cells may first be subject to a range of mutagenic or potentially mutagenic manipulations such as with chemical mutagenic agents, UV light and genetic manipulations with, for example, viruses, electroporation and microinjection of mutagenic genetic material, amongst other procedures. A resulting derivative CEF cell line may produce an altered or derivatised factor having improved or more efficacious properties in maintaining ES cell lines. An additional aspect of this invention extends to such altered or derivatised factors. Derivatised factors where one or more amino acids are deleted, replaced and/or substituted may be produced as described above, or by direct mutagenesis (such as site directed mutagenesis (22)) of the encoding genomic DNA or cDNA followed by polypeptide expression in a suitable host cell. Derivatised factors may also be produced by chemical modification of the polypeptide backbone according to methods well known in the art.

The factor associated with CEF is substantially not secreted or released from the cell as evidenced by the low activity of CEF conditioned medium to maintain ES cells (see Example 1). It is probable, therefore, that the factor is cell or cell-matrix associated. The present

invention, however, extends to a soluble form of the factor prepared by any number of techniques including membrane disruption, cell extraction, physical shearing or other membrane or cell component solubilising procedures. Conveniently, the factor can be sourced from CEE. Alternatively, the factor may be cloned allowing the production of recombinant factor. Cloning may be by any number of procedures including first purifying the factor, ascertaining N-terminal, C-terminal and/or internal amino acid sequences, deducing oligonucleotide probes from these sequences and then screening a suitable chicken cDNA or genomic library. Alternatively, a chicken cDNA or genomic library could be prepared using a suitable expression vector and expression products screened for their ability to maintain ES cells or by reaction with anti-factor polyclonal or monoclonal antibodies. Suitable cloning methods are described, for example, in Sambrook et al (12) which is incorporated therein by reference.

Another aspect of the present invention provides a chick-derived stem cell factor having one or more of the following properties:

(i) a molecular weight of approximately 15,000-35,000 daltons and preferably 20,000-30,000 daltons as determined by gel filtration chromatography;

(ii) elutes from a 10-90% v/v acetonitrile linear gradient during reverse-phase C_8 HPLC at approximately 49% acetonitrile; and

(iii) has an amino acid sequence in the N-terminal region of Xaa^1 Pro Val Ala Gly Tyr Xaa^2 (SEQ ID No 6);
wherein Xaa^1 represents four unknown N-terminal amino acids and Xaa^2 represents the remaining amino acids of the polypeptide.

Additionally, the chick-derived stem cell factor:

(i) is substantially non-reactive to antibodies directed against recombinant mouse leukaemia inhibitory factor (LIF); and

(ii) is encoded by a gene having a nucleotide sequence in its coding regions of less than approximately 70% homology averaged over the length of the cDNA molecule when compared to the cDNA sequence encoding mouse LIF.

Put in alternative terms, the chick-derived stem cell factor comprises a protein having a molecular weight as determined by gel filtration chromatography of approximately 15,000-35,000 daltons and preferably 20,000-30,000 daltons, has an amino acid sequence in the N-terminal region of Xaa^1 Pro Val Ala Gly Tyr Xaa^2 (SEQ ID No 6), is derivable from CEF and CEE and is capable of maintaining ES cell *in vitro* without substantial differentiation in a dose dependant manner. The chick-derived stem cell factor may further be characterized by having an approximate molecular weight on 12.5% SDS-PAGE of about 50,000 daltons. The difference in molecular weight between gel filtration chromatography and SDS-PAGE is presumably due to the effects of glycosylation.

Accordingly, another aspect of the present invention provides an isolated chick-derived ES cell factor capable of maintaining *in vitro* animal ES cells as herein described in the substantially undifferentiated state. By "isolated" is meant a preparation of the factor as described above and extends to recombinant and chemically synthetic forms thereof. The isolated factor is preferably biologically pure e.g. 30-80% or greater than 90% purity relative to other chicken derived components as determined by weight, or other convenient means. However, the present invention also extends to conditioned medium or supernatant fluid containing the ES cell factor whether produced by recombinant cells over-expressing the factor (for example, by expressing DNA encoding the factor in suitable host cells) or by non-recombinant cells, but substantially devoid of cells.

Although the chicken ES cell factor has some activity in common with LIF, there does not appear to be any genetic similarity between the two factors, and if any genetic similarity exists, it would be at a level of less than 70% genetic homology. Without wishing to be bound by any mechanism of action, the factor may act through the LIF receptor.

The capacity to maintain ES cells, stem cells and haemopoietic cells in culture allows various genetic manipulations to be carried out, such as the introduction of foreign DNA for purposes of gene therapy or the production of transgenic animals possessing desirable phenotypes.

The present invention is further described by reference to the following non-limiting Figures and Example.

In the Figures:

Figure 1 is a graphical representation showing the growth of STO cells.

Figure 2 is a photographic representation showing the effect of feeders on mouse ES (D3) clones. A. Inactivated CEF feeders (200x); B. Inactivated STO feeders (200x); C. Gelatin (100x).

Growth of mouse ES colonies after 9 days on

A Inactivated CEF feeders (200 X mag)

B Inactivated STO feeders (200 X mag)

C Gelatin (100 X mag)

Figure 3 is a photographic representation showing the detection of the LIF gene in mouse but not chicken.

A BRL DNA molecular weight markers

B 90/2 WL genomic DNA digested with BamHI (10μg)

C 90/35 LD genomic DNA digested with BamHI (10μg)

D 90/3 Australorp DNA digested with BamHI (10μg)

E Mouse genomic DNA digested with BamHI (10μg)

F 720bp hLIF fragment (100pg)

D PolyA$^+$ RNA from STOs (4.2µg)

E PolyA$^+$ RNA from CEFs (5.1µg)

F Promega RNA ladder (5µg)

Figure 5 is a schematic representation of the primers used in the PCR amplification of RT RNA.

Figure 6 is a photographic representation showing a PCR product from STO RT RNA of mouse but absent in chicken.

A BRL M$_r$ Markers

B no DNA control

C PCR product from RT RNA from STO cells

D no primer control

E RT RNA from CEF cells

F RT RNA from pBluescript/hLIF

Figure 7a is a graphical representation of a RP HPLC separation profile of chick-embryonic extract with activity in the ES colony assay represented as a bar graph under the corresponding peaks.

Figure 7b is a graphical representation showing a bar graph of the results of a DA-1a assay of pooled fraction 1V (■) from Figure 7a compared with CEE (▩) and TGFβ (▩).

Figure 8 is a graphical representation of three RP HPLC profiles measured at 220 nm of proteins eluted under different conditions of cation exchange. The hatched area under the peaks indicates the regions containing activity in the DA-1a assay. I. 0.1 M NaCl; II. 0.3 M NaCl; III. 0.5 M NaCl.

Figure 8 is a graphical representation of three RP HPLC profiles measured at 220 nm of proteins eluted under different conditions of cation exchange. The hatched area under the peaks indicates the regions containing activity in the DA-1a assay. I. 0.1 M NaCl; II. 0.3 M NaCl; III. 0.5 M NaCl.

Figure 9 is a graphical representation of Microbore RP HPLC separated 220 nm profile of fractions 37-38 from 0.1 M NaCl salt step elution. Hatched area represents fractions containing factor activity as seen by the DA-1a and ES colony assay.

Figure 10 is a photographic representation showing a 12.5% w/v SDS-PAGE stained with silver nitrate. Lane 1, Fraction 24 Microbore; Lane 2, Fraction 25 Microbore; Lane 3, Sigma Low Molecular Weight Markers; Lane 4, Fraction 23 Microbore; Lane 5, Fraction 26 Microbore.

ABBREVIATIONS

CEE	chicken embryo extract
CEF	chicken embryonic fibroblast
cDNA	complementary DNA
cES	chicken embryonic stem cells
CO_2	carbon dioxide
DMEM	dulbeccos modified eagles medium
DNA	dioxyribonucleic acid
FCS	foetal calf serum
ICM	inner cell mass
HCl	hydrochloric acid
hLIF	human LIF
LIF	leukaemia inhibitory factor
M	molar
Mr	apparent molecular weight

10 dilution into fresh medium (DMEM) supplemented with 10% v/v NCS or 10% v/v FCS at 37°C, 90% humidity and 5% v/v CO_2

Mouse Embryonic Stem Cells

Mouse ES cells (D3 A) (14) were grown on a confluent layer of mitomycin C (Sigma) inactivated STO cells (15) in ES media, which comprises DMEM supplemented by 5% v/v FCS, 10% v/v NCS and 10^{-4}M β-mercaptoethanol (15). Under these conditions, colonies of stem cells formed which contained small cells with a large nucleus and minimal cytoplasm. The nuclei contained one or more dark nucleolar structures. The cells packed tightly together in small nests from which it was difficult to discern the individual component cells. Borders of these colonies were not as discreet as with colonies grown on primary embryonic fibroblasts but tend to mimic the morphology of colonies grown in the presence of LIF only. Plates were split using standard trypsinisation procedure. After several days, small colonies could be discerned.

1. Growth rate of D3 A cells on STO feeders

Crude estimates of growth rates made by determining time for the culture to reach stationary phase after trypsinisation splitting would suggest that under these conditions growth was rapid. Stationary phase was reached with cells within 2-3 days of splitting, to give an initial cell density of 2×10^4 cells/cm^2.

2. D3 A morphology on STO feeders

ES cell morphology is maintained when cells are routinely subcultured every 2-3 days and refed as the media is exhausted. If these conditions are not maintained, differentiation occurs within 6-8 days.

3. Identification and characterisation of ES cells

Evidence for pluripotency of mouse ES cells is documented by their capacity to differentiate *in vivo* into various cell types of all three primary germ layers. Identification of ES cells *in*

vitro is by differentiation in the absence of feeder cells, normal karyotype and alkaline phosphatase activity (18). Mouse ES cells as well as cells of the ICM are known to show high levels of alkaline phosphatase activity. This activity declines during progressive differentiation, resulting in low alkaline phosphatase levels in somatic differentiated cells (9). Feeder cells do not have alkaline phosphatase activity.

4. D3 A morphology on CEF feeders

To determine the effect of CEF feeders of D3 A morphology, the following pilot was set up.

A 12 well Costar tissue culture plate was gelatin (porcine skin, Sigma) coated. CEF and STO cells were inactivated and plated at 2 x 10^5 cells/well. A row of inactivated STO cells were plated as a positive control for normal ES maintenance with a row of wells coated with gelatin but without feeders as a negative control for lack of maintenance (see Table 1).

TABLE 1

Arrangement of feeder cells

WELLS 1-4	
A	2 X 10^5/well STO cells on gelatin
B	2 x 10^5 well CEF cells on gelatin
C	Gelatin only

D3 A cells were trypsinised from a confluent plate grown on STO feeders and plated at approximately 10^6 cells/well in the first well for each row. Standard ES media was used as described above. Cells were split 1 in 4 using 0.125% w/v trypsin every 3 days and seeded into the next well of the same row. Cells from the latest passage were fed daily with fresh ES medium. Cell numbers were counted prior to each split. Representative fields of cells

NaCl	sodium chloride
NCS	newborn calf serum
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGC	primordial germ cell
RT PCR	reverse transcribed PCR
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TFA	trifluoroacetic acid
TGFβ	transforming growth factor β
TB	transcription buffer
U	unit
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) thiazolyl blue
mLIF	mouse LIF
rLIF	recombinant LIF
RP HPLC	reverse phase high pressure liquid chromatography
UV	ultra violet

EXAMPLE 1

Growth of Primary Chicken Embryonic Fibroblasts (CEF) as 37°C for extended periods

CEF's are a primary cell line with a finite life in culture. The primary cell line was derived by trypsinisation of whole tissue from eight to ten day White Leghorn embryos, following the method of Freshney (13). This batch of cells was called CEF. Cells were initially subcultured every four to five days by a 1 in 10 dilution into fresh CEF medium (DMEM) supplemented with 10% NCS, 2% chicken serum (CSL), 0.01U/ml penicillin and 50Ug/ml streptomycin (CSL). Cells were incubated at 37°C, 5% CO_2 and 95% humidity.

Growth of STO (feeder) cells at 37°C for extended periods

STO cells (12) are thioguanine and ouabain resistant and are a fibroblast line from a SIM mouse (13). Cells were subcultured every four to five days from a confluent dish by a 1 in

were photographed prior to each split as a record of morphology. At the end of the experiment, cells from the final passage were fixed and stained for alkaline phosphatase.

No difference was observed between well 1 in all rows after 3 days growth, however after day 6 in well 2, the cell count on gelatin was a log less than D3 A cells on both CEF and STO wells. Colonies on gelatin were still basically stem cells, however, on the border of each colony, a differentiating cell morphology was seen. ES colonies on STO and CEF feeders had the same morphology. By day 9 in well 3, cells on gelatin have a dramatically different morphology compared with cells from wells 1 and 2. The majority of cells have differentiated into a monolayer of oriented endoderm-like and neuronal-like cells. ES colonies on STO and CEF still maintain the discreet ES colony morphology (see Figure 2).

Cells having a high alkaline phosphatase activity stain pink after fixation and treatment with the Sigma alkaline phosphatase assay kit. Cells with low activity do not stain or are stained very poorly.

Strong alkaline phosphatase staining was observed on all ES cell-like colonies grown on STO and CEF feeder layers. Residual colonies of stem cells on gelatin plates stained pink, however, feeder cells and the mass of monolayer cells (see below) did not stain at all.

LIF HOMOLOGY IN CHICKEN

The results obtained above indicated the presence of a factor or factors in CEF's which enables the maintenance of mES cells in culture. To determine whether this factor is homologous to LIF, experiments were conducted to see if a homologous sequence to LIF could be identified in chicken genomic DNA.

Using the hybridisation conditions described by Willson (10), LIF homologies as low as 74% at the nucleotide level (between mouse and sheep) should be detectable. Birds diverged from mammals an estimated 200 million years ago and such a large evolutionary distance would

suggest that bird genes would be less than 70% homologous to mammalian sequences (across a gene sequence), unless conservation of function has maintained those sequences. A functional LIF homology could be present in chickens with only the functional regions in the molecule, such as the receptor binding site, being conserved. If this region is sufficiently large, then it may be possible, using a full length heterologous LIF probe, to detect a homology using lower stringency conditions.

To test this, both genomic DNA from chicken blood and polyA$^+$ mRNA from CEF's were screened. Mouse genomic DNA was isolated from mouse tails and polyA$^+$ mRNA from STO cells was screened for comparison.

1. Southern Blot Analysis

Aliquots of BamH1 restriction-enzyme-digested chicken and mouse genomic DNA's (10µg) were electrophoresed in 0.8% w/v agarose gels, blotted onto Hybond N$^+$ membrane and prehybridised and hybridised in 6 x SSC, 5 x Denhardts, 0.5% w/v SDS and 100 µg/ml sheared Salmon Sperm DNA. The hybridisation probe (720bp Xho I fragment from pXM.6R which contains the human LIF cDNA (8)) was generated by random labelling using a Bresatec Gigaprime Kit, and used at ~10^7 cpm/ml. Filters were washed in 2 x SSC, 0.1% w/v SDS at either 50, 55, 60 and 65°C, prior to autoradiography. At 65°C (Figure 3), a unique 3kb band was detected in mouse genomic DNA using a full length human LIF cDNA fragment probe. At equivalent loadings, no bands were detected in chicken genomic DNA at this stringency. This result would indicate that if a LIF homology was present, it would have to have less than 70% homology to the human LIF clone. Hybridisation at lower stringencies did not reveal hybridisation of a unique band.

2. Northern Blot Analysis

Total RNA from confluent plates of CEF's and STO cell lines was isolated by single step extraction with an acid guanidinium thiocyanate-phenol-chloroform (16). PolyA$^+$ mRNA was isolated from total RNA using the Promega Poly A Tract mRNA Isolation System IV (Cat

number 25310). The polyA$^+$ mRNA was concentrated and 5µg RNA/track was electrophoresed in 1% formaldehyde/agarose, blotted onto Hybond N$^+$ membrane and prehybridised and hybridised in 50% v/v deionised formamide, 5 x SSC, 1 x PE and 150 µg/ml sheared salmon sperm DNA. The hybridisation probe was made by ^{32}P labelled transcription of a 720bp XhoI fragment from the human LIF cDNA clone in a pBluescript vector using the T_3 promoter of the vector. The transcript was made using a Promega Riboprobe Kit and used at ~10^7 cpm/ml. Filters were washed in 2 x SSC, 0.1% w/v SDS at 65°C, prior to autoradiography. Using the hybridisation conditions described above, a ~700 bp band was detected in STO polyA$^+$ mRNA but no hybridisation was evident for CEF polyA$^+$ RNA (see Figure 4). This indicates that under these conditions a RNA species of sufficient homology to human LIF is not present in chickens.

3. PCR amplification of Reverse Transcription (RT) RNA

PCR of RT RNA can be used to amplify genes from different species, if regions of sufficient homology can be determined. By comparing the DNA sequence of mouse and human, regions of high conservation between the two were selected and complementary oligonucleotides designed (see Figure 5).

RNA was prepared (11) from confluent plates of COS-1, STO and CEF cells, grown as previously described. RNA transcripts were generated from the T7 polymerase promoter in pBluescript II SK$^+$/hLIF 720bp XhoI clone to provide a positive control for amplification.

Reverse transcription of the RNA was achieved using 2 µg RNA, 1 x TB, 6U RNasin (Promega), dNTP (Pharmacia) and 10-20 Units of AMV reverse transcriptase. The reaction was incubated at 37°C for 2 hours prior to DNAse treatment, phenol/chloroform extraction and ethanol precipitation. This cDNA provided the template for PCR amplification.

RT RNA was amplified using the primers RILIF, H3 LIF, (Figure 5) Universal and Reverse primers (17), at 20pmol/reaction in 0.2mM dNTP (Pharmacia Ultrapure dNTP set Cat#27-

2035) Promega 1 x Taq polymerase buffer, 2.5U Taq polymerase (Promega), 10pg template, in a reaction volume of 50 µl. Reactions were overlain with 50 µl Mineral oil and heated to 96°C prior to 25 cycles of 1 minute at 96°C, 20 seconds at 45°C, 1 minute 20 seconds at 72°C in a Thermocycler (Cherlyn Electronics, Cambridge England Cat # IHB 2024). A 10µl aliquot of this reaction was then reamplified using the conditions described above and 10% of the reaction electrophoresed on a 0.8% w/v Agarose/EtBr gel. A 639 bp band was amplified from STO RT RNA after the second round of PCR (see Figure 6). No amplified product was detected from the other samples.

SCREENING FOR CHICKEN ES FACTOR IN CULTURE MEDIUM

Conditioned Medium (CM) was prepared as follows: Corning tissue culture plates (5 x 10cm) were seeded with either STO or CEF cells at 5 x 10^5 cells/plate in 12 ml of DMEM, 10% v/v NCS and 2% v/v CS. After 3 days, the medium was taken off the cells, spun to remove cellular debris, filtered through 0.45µ filters and stored at -20°C.

Costar 12 well tissue culture plates were prepared with and without feeders as described above. Mouse ES cells, D3 A, were seeded at a range of concentrations in different media as shown below. D3 A cells were also plated onto STO and CEF feeders in the presence of the various media to ensure that factors in the CM did not alter growth patterns. Cells were split 3 times over a period of 9 days into each of the media and the microscopic morphology recorded at Day 7. In the Table 2 below, the increasing number of ES colonies is indicated by an increasing number of "+" signs. A "-" sign indicates the present of differentiated cells.

Table 2 summarises the comparison of the ES colony morphology after 7 days growth in the different conditioned media. The results show that within 7 days, ES cells differentiate in the absence of feeders or conditioned medium, whereas ES cells grown in the presence of CEF/STO feeders or LIF, are maintained. The proliferation of ES cells on LIF, in the absence of feeders, shows that the ES cells used in these experiments are responsive to LIF and that the conditioned medium does not contain specific inhibitors of their growth. In the absence

of feeders, STO CM supports the growth of ES cells, although a small percentage of cells differentiate. ES cells grown in CEF CM, on the other hand, proliferate but contain a high percentage of differentiated cells indicating that only a minimal amount of factor is soluble.

TABLE 2
Morphology of ES cells in Conditioned media

CONDITIONED MEDIA (CM)	COMPARISON OF ES MORPHOLOGY AT DAY 7			
	+ Feeders STO	+ Feeders CEF	- Feeders	- Feeders/ +LIF
No CM	+	++	-	+++
STO CM	+++	+++	+++/-	+++
CEF CM	++++	++++	++/----	+++

EXAMPLE 2

PURIFICATION OF CHICK-DERIVED ES CELL FACTOR

I. SOURCE MATERIAL

1. Materials and Methods

1. Preparation of material for isolation of the chicken factor

An alternative source of chicken embryonic material was sought in order to provide a greater source of material from which to purify the chick-derived ES cell factor.

Acid extracted 10 to 12 day chick embryos were tested for activity using the mouse embryonic stem cell lines MBL5 (16), E14 (23) and the DA-1a assay (19). The extract was also tested for the presence of TGFβ, which is often present in high amounts in embryonic tissue and is an inhibitor of the DA-1a Assay. This can be tested using TGFβ sensitive Mink Lung cells(CCL64).

2. Preparation of Chick Embryonic Extract (CEE)

CEE were prepared from Day 10-12 incubated E12 chick embryos. Eggs were first rinsed in 70% v/v ethanol. Embryos were then accessed through the shell and immediately the head was separated from the body. Bodies were washed in phosphate buffered saline (PBS), then roughly macerated and weighed. To each gram of wet weight tissue was added 0.1% v/v trifluoroacetic acid (TFA). The acidified tissue was then blended using a Polytron blender for 3 minute. The homogenate was aliquoted and stored at -20°C. Upon thawing, the extract was centrifuged at 8,000rpm for 30 minutes at 4°C in a Sorvall RC-5B. The supernatant was decanted and stored at 4°C.

3. ES Colony Assay

MBL5 or E14 cells were plated 1000/well in Costar 48-well plates. To each well was added the appropriate dilution of CEE or fractionated CEE to give a range of concentration of CEE in 1X ES medium (Dulbecco's modified Eagles media (DMEM F-12) (ICN Flow) supplemented with 15% w/v foetal calf serum (ICN Flow or Imperial), 0.12% w/v sodium bicarbonate, 2mM glutamine, 0.01U/ml penicillin and 50ug/ml streptomycin (ICN Flow)). In addition, controlled cultures containing 10uM 2-mercaptoethanol and 1000U/ml recombinant mouse LIF (rmLIF) were added to the growth medium. Cells were incubated at 37°C, 5% CO_2 and 95% humidity for 5 days. Growth medium was removed and the cells stained with Leishmans stain (BDH) or by the alkaline phosphatase assay. ES colonies were scored qualitatively as dark staining clumps of cells or as alkaline phosphatase positive colonies, respectively.

4. DA-1a Assay

DA-1a cells are a LIF-dependent cell line derived from mouse leukemia cells. The cells were maintained in DA-1a medium (1X RPMI 1640 medium with 2g/l sodium bicarbonate, without glutamine (ICN Flow) supplemented with 10% v/v foetal calf serum (ICN Flow or Imperial), 0.12% w/v sodium bicarbonate, 2mM glutamine, 0.01U/ml penicillin and 50ug/ml streptomycin (ICN Flow) and 10U/ml of mouse recombinant LIF. Prior to assay, the cells were washed 3 times with 50ml of saline solution, containing 2% v/v FCS without LIF and

centrifuged at 1,000rpm for 10 minutes. After the final wash, the cells were resuspended in RPMI medium supplemented with 10% v/v FCS (without LIF) to a final concentration of $2X10^5$ cells/ml. Assays were carried out using a 96 well flat bottomed plate containing 50ul of cell suspension and factor per well. The plates were incubated for three days prior to staining with MTT (see 6 below).

5. TGFβ Assay (20)

Growth of Mink Lung cells (CCL64) is strongly inhibited by TGFβ. Cells were grown at 37°C, 5% CO_2 and 95% humidity in DMEM-F12 supplemented with 10% v/v FCS, 0.12% w/v sodium bicarbonate, 2mM glutamine, 0.01U/ml penicillin and 50ug/ml streptomycin. For assay, 1000 cells per well in a 96 well flat bottomed plate were added in a volume of 50ul at a concentration of $2X10^4$ cells/ml and allowed to attach for 4 hours prior to the addition of factor. Factor to be assayed was in 50ul volume and a 2X serial dilution (in culture medium) of each sample were made to assess activity. Plates were incubated for 5 days prior to staining with MTT (see 6. below).

6. MTT Staining to measure proliferative cell growth

An aliquot of 10ul of a 5mg/ml solution of MTT (Sigma) dissolved in PBS and filter sterilised was added to each well of a proliferative assay. The MTT solution was then incubated with the cells for 4 hours at 37°C and 5% CO_2. The crystals which formed on the proliferating cells were then dissolved in 150ul of isopropanol, 0.04N HCl containing 1% v/v Triton X-100. Absorbency at 570nm is measured using an Anthos Labtec plate reader. Maximal absorbance is a measure of highly proliferative cells.

7. Alkaline phosphatase staining of embryonic stem cells

A diagnostic feature of the undifferentiated state of ES cells is the positive reaction in the alkaline phosphatase assay. As cells differentiate, their ability to be stained by this method is lost. This enables accurate assessment of the state of ES cells. The method used is

essentially that described in the Sigma procedure number 86 (18) using the Alkaline Phosphatase Leukocyte Assay except that staining was performed in the assay wells. Half the volume of the medium was replaced with Citrate-Acetone-Formaldehyde Solution (CAFS), then removed. Cells were fixed in 100% CAFS for 30 secs. The cells were then rinsed in water prior to the addition of alkaline dye mix. After incubation in the dye mix for a maximum of 15 minutes the dye mix is replaced with water for at least 2 minutes to stop the reaction before air drying.

2. Results

CEE showed no significant proliferative effect in the DA-1a assay (see Figure 7b). This could be due to an absence of factor/s which interact with the LIF receptors on the DA-1a cells or be a result of other inhibitory factors. Results from the Mink Lung assay indicated that the CEE contained the equivalent of $\geq$ 100pg active TGFβ, which is sufficient to inhibit the DA-1a assay (see Figure 7b).

EXAMPLE 3

PURIFICATION OF CHICK-DERIVED ES CELL FACTOR.

II. PROTOCOL FOR PURIFICATION FROM CHICKEN EMBRYONIC EXTRACT

A number of purification procedures were initiated in order to characterise the activity in CEE. These included small-scale ion exchange chromatography and RP HPLC to determine the elution profile and the feasibility of maintaining activity of the factor during these steps. Gel filtration of partially purified extract was performed to determine the relative molecular weight of the factor.

1. Materials and Methods

1. <u>Purification of CEE by reverse phase (RP) HPLC</u>

The flow chart (Scheme I) below shows a pilot purification of CEE by RP HPLC. One millilitre of clarified CEE was acidified with 0.1% v/v TFA (pH~2.5) and bound to A C-PAK column which had been equilibrated in 10 volumes of 0.1% v/v TFA. Protein was eluted in

10 ml 60% acetonitrile (BDH)/0.05% v/v TFA The percentage of acetonitrile in the eluate was reduced to 20% by the addition of twice the eluate volume of 0.1% v/v TFA. A third of the material was loaded onto a Beckman Ultrapore RPMC C_8 (4.6mmX7.5cm) column, equilibrated in 20% acetonitrile. A 10 to 90% acetonitrile linear gradient was generated over 60 minute and 0.5ml fractions collected. Figure 7 represents the profile from the RP HPLC gradient (measured at 220nm) with the number of ES colonies supported by the activity of each fraction represented as a bar graph. The activity of pooled fractions from the same gradient was measured by the DA-1a assay.

2. Results

The results of the ES colony assay and the DA-1a assay (respectively 7a and 7b) showed that high activity is detected in the fractions eluting at about 46 to 53%, and more precisely about 49% acetonitrile. The ES colony assay detected activity in three consecutive fractions at a dilution of 1/100. Every fourth consecutive fraction from the RP HPLC was pooled prior to assay by DA-1a cells. Activity in the DA-1a assay is detected at a dilution as high as 1/100. This activity is equivalent to 38U/ml mrLIF. No significant activity was detected by either assay in any of the other fractions assayed.

These results indicate that the chick-derived ES cell factor remains active in the buffers used for HPLC and that RP HPLC separates this activity from inhibitory factors present in the crude CEE extract. The single peak of activity eluting at ~49% Acetonitrile (Pooled Fraction IV) indicates that either the factor is a single component or that a number of factors have identical properties under these RP HPLC conditions.

Scheme I

Flow chart for RP HPLC separation

CLARIFIED CEE(1ML)
↓
C-PAK COLUMN (C_{18})
ELUTED AT 60% ACETONITRILE
↓
CEE DILUTED TO 20% ACETONITRILE
↓
RP HPLC (C_8)
ELUTED AT 10-90% ACETONITRILE
↓
ASSAY INDIVIDUAL FRACTIONS
BY ES COLONY ASSAY
AND
POOLED FRACTIONS BY DA-1A ASSAY

EXAMPLE 4

Molecular weight determination of the chick-derived ES cell factor

Gel filtration of Pooled Fraction IV (Example 3) was performed to determine the apparent molecular weight of the factor. This was achieved by gel filtration chromatography at 20% acetonitrile/0.1% v/v TFA at 1ml/minute with a Beckman Ultraspherogel SEC 2000 column. Standard proteins were chromatographed for calibration. The activity of the fractions after gel filtration were measured by the DA-1a assay. Fractions containing activity eluted from the column at 40 to 42 minutes. This would correspond to an apparent molecular weight of 20,000 to 30,000 daltons under the above conditions and more particularly 25,000 to 27,000 daltons.

EXAMPLE 5

Further purification of chick-derived ES cell factor

1. Cation exchange Chromatography of CEE

Preliminary purification of CEE by RP HPLC showed a complex of proteins in fractions containing factor activity. As activity is maintained after RP HPLC this provides a suitable second stage purification. Cation exchange chromatography was chosen as an initial step due to the maintenance of activity of the initial extract at an acid pH.

Two purification schemes were used to separate CEE by cation exchange in a 100mM phosphate buffer pH 6.0 (Scheme II flowchart). The first involved a three step salt elution at 0.1, 0.3 and 0.5M NaCl followed by RP HPLC (Figure 8a). This broad step salt elution was used to assess the purification potential of cation exchange. A pH of 6.0 in 100mM phosphate buffer was chosen due to the buffering potential of that molarity phosphate and the corresponding acidity of the extract. A linear salt gradient from 0-0.5M NaCl on Mono S resin, followed by RP HPLC, was chosen to assess the actual elution of the protein activity.

A salt step gradient was applied to a Pharmacia 1ml SP Sepharose HP column equilibrated in 100mM Phosphate pH 6.0. One mL of clarified CEE was bound to the column in equilibration buffer. Three column volumes of equilibration buffer was passed over the column. Proteins were eluted by the sequential addition of three volumes of each salt concentration of 0.1, 0.3 and 0.5M NaCl in the equilibration buffer. Prior to loading onto the reverse phase column, fractions were acidified to pH 2.5 by the addition of TFA to a final concentration of 0.1%. The fractions were then filtered through a 0.22uM filter to remove any precipitate formed. This material was then bound to a Beckman C_8 RP HPLC column in 10% acetonitrile/0.1% v/v TFA. The protein was eluted from the column at 1ml/minute by a linear increase of acetonitrile of 1.33%/minute over 60 minutes for samples from 0.1 and 0.3M NaCl and by a non-linear gradient for 0.5M NaCl. Fractions containing activity in the DA-1a assay were then further purified and concentrated by eluting proteins from a Microbore RP HPLC using a gradient where the acetonitrile concentration was increased at the rate of 1%/minute. A summary of this purification is shown in the Scheme II flow chart.

Scheme II
Flow chart of purification

CLARIFIED CEE (1ML)	
↓	
BOUND TO SP SEPHAROSE IN 100mM PHOSPHATE pH 6.0	
↓	
ELUTE BY SALT STEP ELUTION 0.1, 0.3 and 0.5M NaCl	
↓	
ACIDIFICATION OF SAMPLE TO 0.1% TFA	
↓	
RP HPLC GRADIENT	-> DA-1a assay
↓	
DA-1A ASSAY POSITIVE FRACTIONS	
↓	
MICROBORE RP HPLC STEP GRADIENT	-> DA-1a assay ->ES colony assay

2. Results

Figure 8 shows the 220 nm absorbance profiles of proteins separated by RP HPLC, from samples eluted after salt elution at 0.1, 0.3 and 0.5M NaCl, respectively. Factor activity, as measured by the DA-1a assay is indicated by the hatched area under the peaks. The profiles indicate that the majority of proteins from the CEE elute at 0.3M NaCl. Activity of the factor eluted at 0.3M NaCl at ~49% acetonitrile, as expected. Activity was also detected in fractions from proteins eluting at 0.1 and 0.5 M NaCl, indicating that the factor is not eluting from the resin at a single salt concentration.

The profile of total CEE proteins eluting from the RP HPLC step would, however, indicate that differential separation was achieved by the 3 salt steps. The spread of factor activity over a range of salt concentrations may be indicative of differentially charged glycosylation of the factor. Activity from each salt step which was bound to the RP column, eluted at the same

percentage acetonitrile. The activity from 0.5M NaCl appears to elute earlier than the other profiles due the fact that a non linear gradient was used to separate this sample. The behaviour of the factor on ion exchange was confirmed by the spread of factor activity found in fractions across a linear (0-0.5M) NaCl gradient eluting CEE from Mono S cation exchange chromatography. Activity was seen to elute continuously from the linear gradient between 0.05M and 0.45M NaCl, with the majority of the activity eluting at between 0.15 and 0.35M NaCl.

Activity eluting at 0.3M NaCl contained too many contaminating proteins for effective elution at this salt concentration despite the presence of high 'factor' activity. Therefore, the factor activity detected in 0.1M and 0.5M NaCl was further purified by Microbore RP HPLC, as these fractions were high in activity.

Figure 9 shows the 220nm absorbance profile of Fractions 37-38 from the 0.1M NaCl RP HPLC elution separated on the Microbore column. The profile would indicate that ~9 protein peaks were further separated from these fractions. The hatched area under the peaks represents the fractions containing factor activity as measured by DA-1a assay and the ES colony assay. The factor activity was detected by ES colony assay and the DA-1a assay as eluting from the microbore column at ~49% acetonitrile as seen in the previous RP HPLC elutions.

EXAMPLE 6

SDS-PAGE &Western blot of CEE using polyclonal antibodies raised against rmLIF

Apart from LIF, DA-1a cells proliferate in the presence of Oncostatin M and IL-3, but are not responsive to CNTF or IL-6. The activity could not be attributed to IL-3 as this molecule has not activity on ES cells. To determine whether the activity found in the fractions containing activity from Figure 10 is LIF related, the samples were separated following 12.5%

w/v SDS PAGE and either silver stained or were blotted against serum from rabbits immunised with rmLIF.

To enable immunoreaction with LIF antisera, proteins were electrophoretically transferred to nitrocellulose(0.45u). The membrane was blocked with 5% w/v BSA in PBS, prior to reaction with a 1/100 dilution of polyclonal antisera raised in rabbits against a Gex-LIF fusion protein (Ab 4855/6). Detection of anti-LIF antibodies was accomplished by a 2 hour incubation of the immunoblot with alkaline phosphatase-conjugated second antibody (Sigma).

Three proteins could be seen at an apparent molecular weight of ~55, ~50 and ~20KDa after silver staining the gel (Figure 11). The 20KDa band cannot be seen in this gel as it has run off the end of the gel. The ~50KDa protein is the major component of the fraction, representing ~85 to 90% of the total protein. It is likely that the 55KDa diffuse band represents differentially glycosylated forms of the 50KDa product. The 20KDa band may represent degradation products of the major 50KDa protein. By comparison to a known amount of rmLIF, an estimate of 40ng/track for the 50KDa band can be made. It appears as a diffuse band typical of glycosylated proteins. Increasing activity in both the DA-1a assay and the ES colony assay corresponds to the increase in concentration of this ~50 KDa band.

Reactivity with the LIF polyclonal antisera was not observed in these fractions with ≥ 40ng chicken factor. A sample of rm LIF electrophoresed as a control, could be detected at greater than 1ng.

The purified factor was subject to N-terminal amino acid sequence analysis as a definitive means of characterising the protein. The first four N-terminal amino acids were masked (due to the presence of glycine). The amino acid sequence of the fifth to ninth N-terminal sequence amino acids was determined . This amino acid sequence is as follows:

Xaa^1 Pro Val Ala Gly Tyr Xaa^2 (SEQ ID No 6)

wherein Xaa[1] represents four unknown N terminal amino acids, and Xaa[2] represents the remainder of the amino acid sequence of the factor.

Table 5 provides a comparison of the N-terminal sequence of the factor with the N-terminal sequence of various animal LIF's

TABLE 5

Comparison of LIF N-terminal sequence from different species to the N-terminus of the Chicken Factor

Mouse	Met	Lys	Val	Leu	Ala	Ala	Gly	Ile	Val	Pro[1]
Rat	Met	Lys	Val	Leu	Ala	Ala	Gly	Ile	Val	Pro[2]
Human	Met	Lys	Val	Leu	Ala	Ala	Gly	Val	Val	Pro[3]
Ovine	Met	Lys	Ile	Leu	Ala	Ala	Gly	Val	Val	Pro[4]
Porcine	Met	Lys	Val	Leu	Ala	Ala	Gly	Val	Val	Pro[5]
Factor	X	X	X	X	Pro	Val	Ala	Gly	Tyr	X

1, SEQ ID No 1; 2, SEQ ID No 2; 3, SEQ ID No 3; 4, SEQ ID No 4; 5, SEQ ID No 5; where X represents an unidentified amino acid.

A comparison of this sequence with the sequences in Table 5 clearly show that the factor is not a LIF molecule. A homology search of this peptide with the complete protein sequence of LIF of various species show that no homology could be found, therefore excluding the possibility that the chicken factor sequence is homologous to an internal LIF sequence.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this

specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Commonwealth Scientific and Industrial Research Organisation
Cancer Research Campaign Technology Limited

(ii) TITLE OF INVENTION: A Method for Maintaining Embryonic Stem Cells and Avian Factor useful for same

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(A)	MEDIUM TYPE:	Floppy disk
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(2) INFORMATION FOR SEQ ID No:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID No:1:

Met	Lys	Val	Leu	Ala	Ala	Gly	Ile	Val	Pro
1				5					10

(2) INFORMATION FOR SEQ ID No:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID No:2:

Met	Lys	Val	Leu	Ala	Ala	Gly	Ile	Val	Pro
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(2) INFORMATION FOR SEQ ID No:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID No:3:

Met	Lys	Val	Leu	Ala	Ala	Gly	Val	Val	Pro
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(2) INFORMATION FOR SEQ ID No:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID No:4:

Met	Lys	Ile	Leu	Ala	Ala	Gly	Val	Val	Pro
1				5					10

(2) INFORMATION FOR SEQ ID No:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID No:5:

Met	Lys	Val	Leu	Ala	Ala	Gly	Val	Val	Pro
1				5					10

(2) INFORMATION FOR SEQ ID No:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID No:6:

Xaa	Pro	Val	Ala	Gly	Tyr	Xaa
1				5		

(2) INFORMATION FOR SEQ ID No:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID No:7:

CTAGAATTCC CATAATGAAG G 21

(2) INFORMATION FOR SEQ ID No:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID No:8:

GTAAAGGTTA GAAGGCCTGG GCC 23

CLAIMS:

1. A method for maintaining *in vitro* animal embryonic stem (ES) cells without substantial differentiation said method comprising culturing said ES cells in the presence of a feeder layer comprising chicken embryonic fibroblasts.

2. A method according to claim 1 wherein the animal ES cells are derived from animals selected from the group consisting of humans, livestock animals, companion animals, laboratory test animals, wild animals, poultry, game birds, caged birds and wild birds.

3. A method according to claim 1 wherein the animal ES cells are derived from mice, rats, chickens, pigs, sheep and cattle.

4. A method according to claim 1 wherein the chicken embryonic fibroblast layer is a confluent monolayer comprising fibroblastoid cells.

5. A method according to claim 4 wherein the chicken embryonic cells are first mutagenized by one or more of a chemical mutagenic agent, UV light or genetic manipulation prior to forming the confluent monolayer.

6. A method according to claim 1 wherein said chicken embryonic fibroblasts contain a cell or cell-matrix associated ES cell factor.

7. A method for maintaining *in vitro* animal ES cells without substantial differentiation, said method comprising culturing said ES cells under appropriate conditions and in the presence of chicken-derived ES cell factor.

8. A method according to claim 7 wherein the chicken-derived ES cell factor is derived from chicken embryonic fibroblasts.

9. A method according to claim 7 wherein the chicken-derived ES cell factor is derived from chicken embryonic extract.

10. A method according to claim 7 wherein said factor is present in conditioned media from cells which express the factor.

11. A method according to claim 7 wherein the animal ES cells are derived from animals selected from the group consisting of humans, livestock animals, laboratory test animals, wild animals, poultry, game birds, caged birds and wild birds.

12. A method according to claim 7 wherein the animal ES cells are derived from mice, rats, chickens, pigs, sheep and cattle.

13. A method according to claim 7 wherein the chick-derived stem cell factor has one or more of the following characteristics:

(i) a molecular weight of approximately 15,000-35,000 daltons as determined by gel filtration chromatography;

(ii) elutes from a 10-90% v/v acetonitrile linear gradient during reverse-phase HPLC on a C_8 column at approximately 46 to 53% acetonitrile; and

(iii) has an amino acid sequence in the N-terminal region of Xaa^1 Pro Val Ala Gly Tyr Xaa^2 (SEQ ID No 6);

wherein Xaa^1 represents four unknown N-terminal amino acids and Xaa^2 represents the remaining amino acids of the polypeptide.

14. A method according to claim 13 wherein the chick-derived stem cell factor:

(i) is substantially non-reactive to polyclonal antibodies to recombinant mouse leukaemia inhibitory factor (LIF); and

(ii) is encoded by a gene having a nucleotide sequence in its coding regions of less than approximately 70% homology averaged over the length of the cDNA molecule when compared to the cDNA sequence encoding mouse LIF.

15. A method according to claim 13, wherein the chick-derived factor has a molecular weight on 12.5% SDS-PAGE of about 50,000 daltons.

16. An isolated chick-derived ES stem cell factor comprising a protein having a molecular weight as determined by gel filtration chromatography of approximately 20,000 to 30,000 daltons, has an amino acid sequence in the N-terminal region of Xaa^1 Pro Val Ala Gly Tyr Xaa^2, wherein Xaa^1 and Xaa^2 are as previously defined, is derivable from chicken embryonic fibroblasts and from chick embryo extracts and is capable of maintaining ES cells *in vitro* without substantial differentiation.

17. An isolated chick-derived ES stem cell factor according to claim 16 further characterised by eluting from a 10-90% v/v acetonitrile linear gradient during reverse phase HPLC C_8 at approximately 46 to 53% acetonitrile.

18. An isolated chick-derived ES stem cell factor according to claim 16 or 17 further characterised by being substantially non-reactive to polyclonal antibodies to recombinant mouse LIF and being encoded by a gene having a nucleotide sequence in its coding regions of less than approximately 70% homology averaged over the length of the molecule when compared to the cDNA sequence encoding mouse LIF.

19. An isolated chick-derived ES stem cell factor according to claim 16 or 17 which has a molecular weight on 12.5% SDS-PAGE of 50,000 daltons.

20. A non-human animal derived from ES cells cultured in the presence of a feeder layer comprising chicken embryonic fibroblasts.

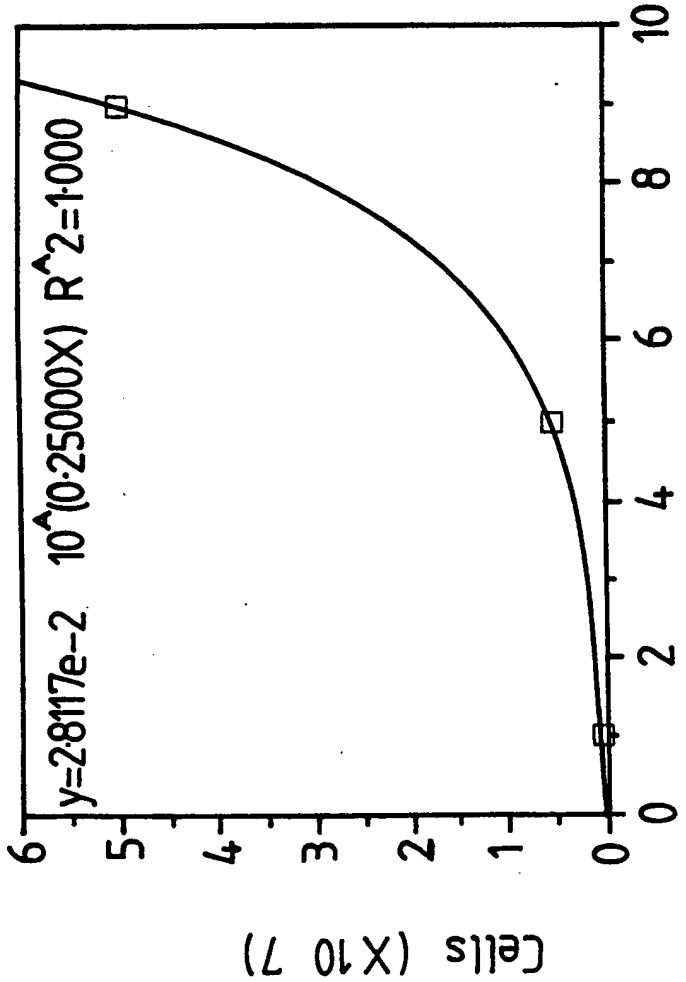
21. A non-human animal derived from ES cells cultured in the presence of chick-derived ES stem cell factor.

22. A non-human animal according to claim 20 or 21 wherein said animal is a chicken, mouse, rat, sheep, pig or cow.

23. A non-human animal according to claim 20 or 21 wherein said animal is a transgenic animal derived from genetically manipulated ES cells.

24. A method for maintaining human or animal primordial germ cells, haemopoietic stem cells, or cell lineage stem cells in culture without substantial differentiation said method comprising culturing said cells in the presence of a feeder layer comprising chicken embryonic fibroblasts or a chicken-derived ES cell factor, optionally in association with one or more cytokine factors.

25. A cell maintained according to the method of claim 22.



Growth curve for STO cells

FIG 1

FIG 2A

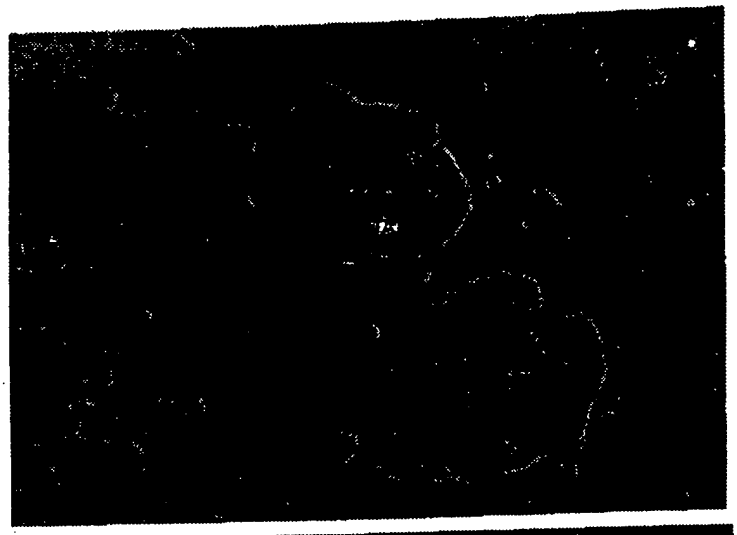


FIG 2B

FIG 2C

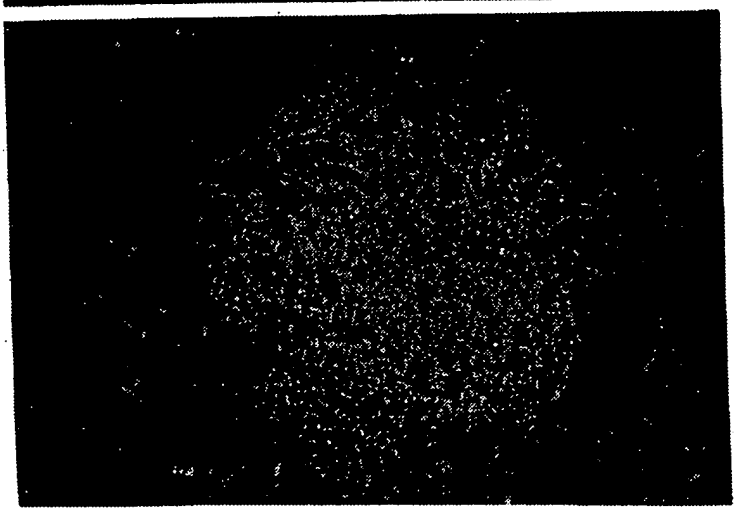


FIG 3

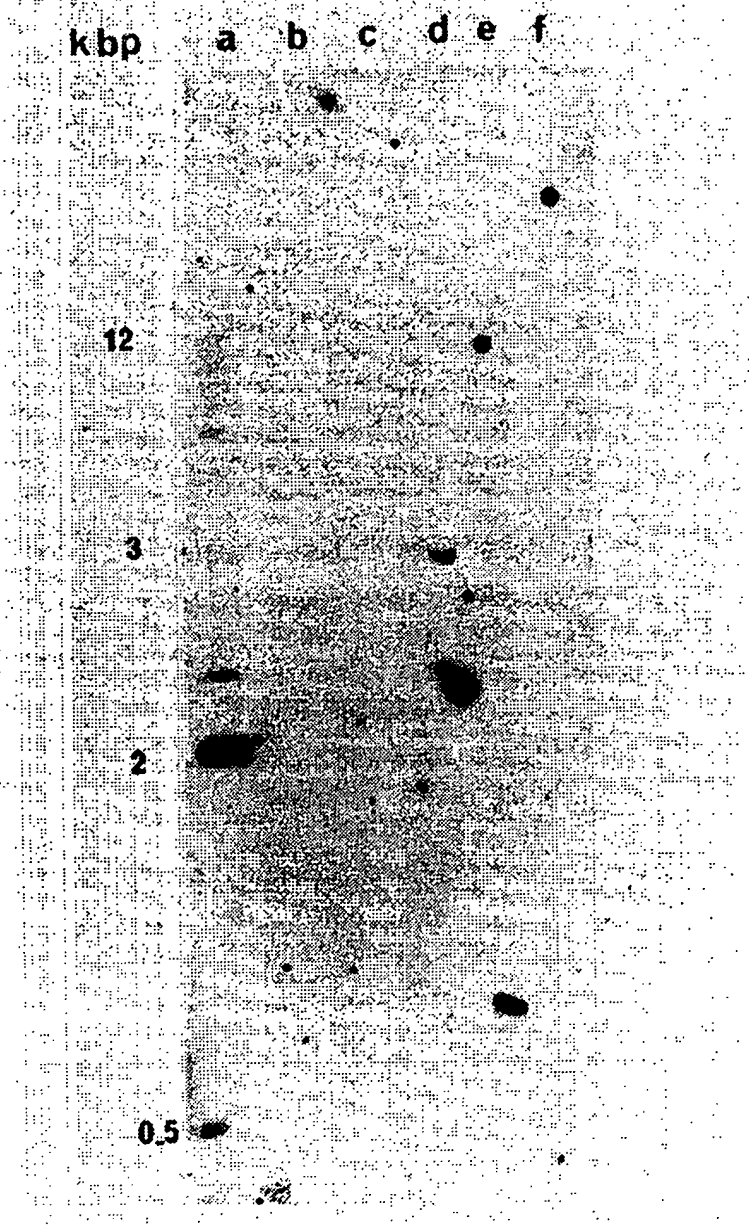


FIG 4

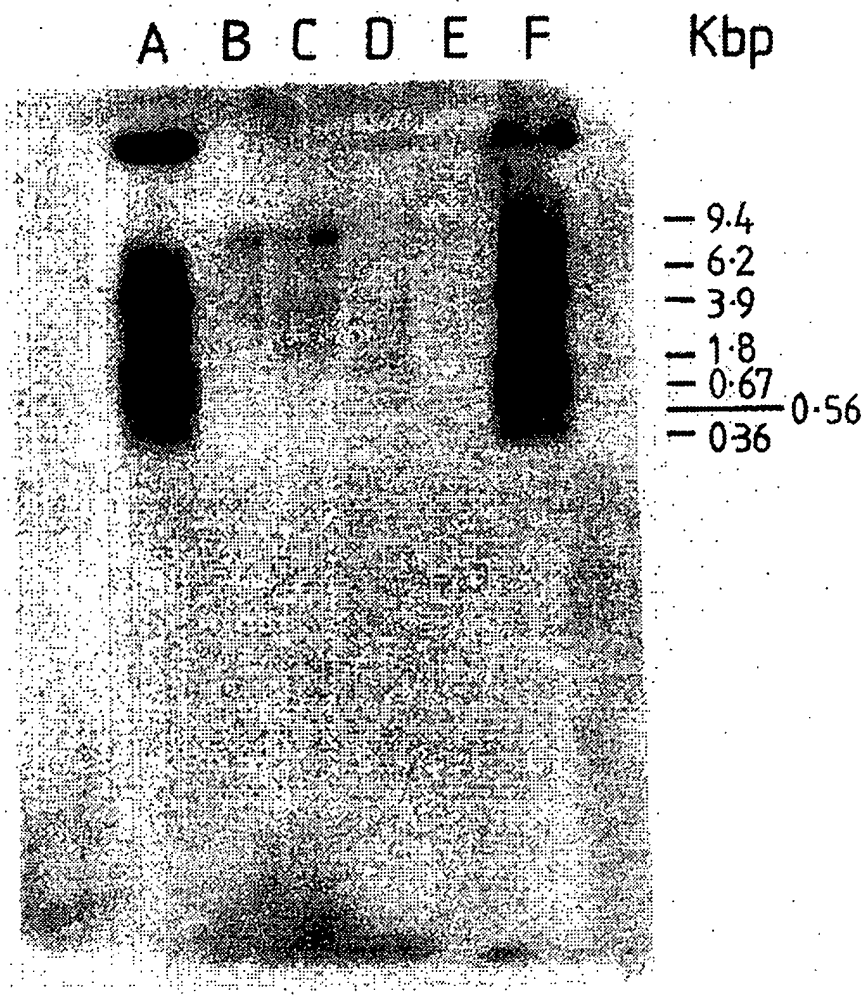


FIGURE 5

RI LIF

5' 3'

EcoRI

CTAG AATTCCCATAATG.AAG.G (SEQ ID No 7)

39* 51*

H3 LIF

5' 3'

HindIII

CTA AAGGTTAGAAGGCCTGGGCC (SEQ ID No 8)

653* 639*

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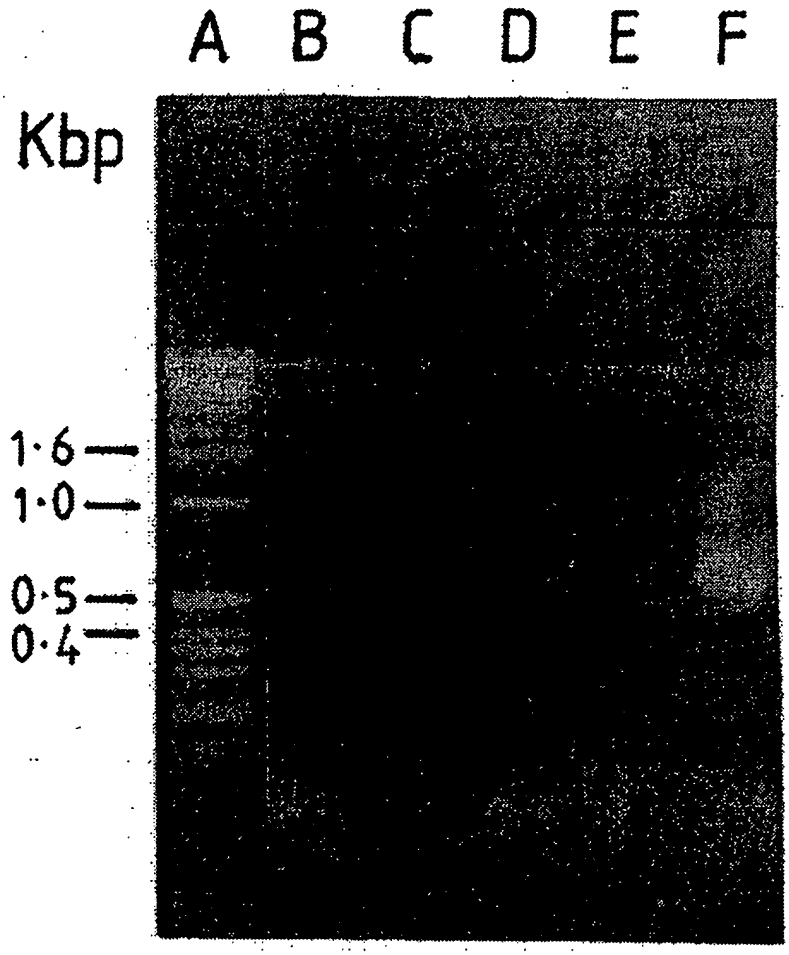
* from published human LIF sequence Moreau et al 1988 [8]

FIG 6



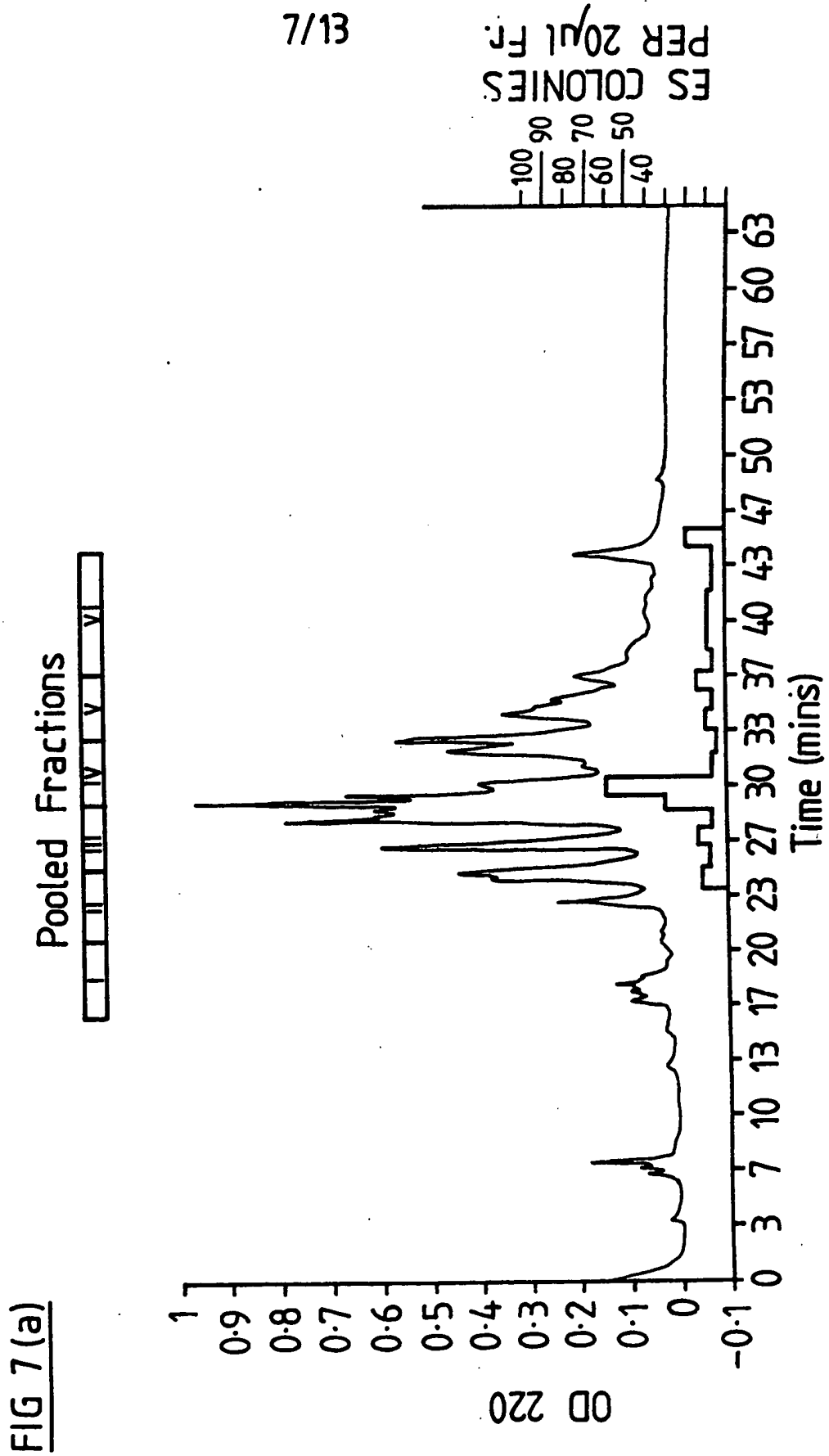

ES COLONIES
PER 20µl Fr.
100
80
60
40
90
70
50
Pooled Fractions
I
II
III
IV
V
VI
OD 220
1
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
-0·1
0
3
7
10
13
17
20
23
27
30
33
37
40
43
47
50
53
57
60
63
Time (mins)

FIG 7(a)

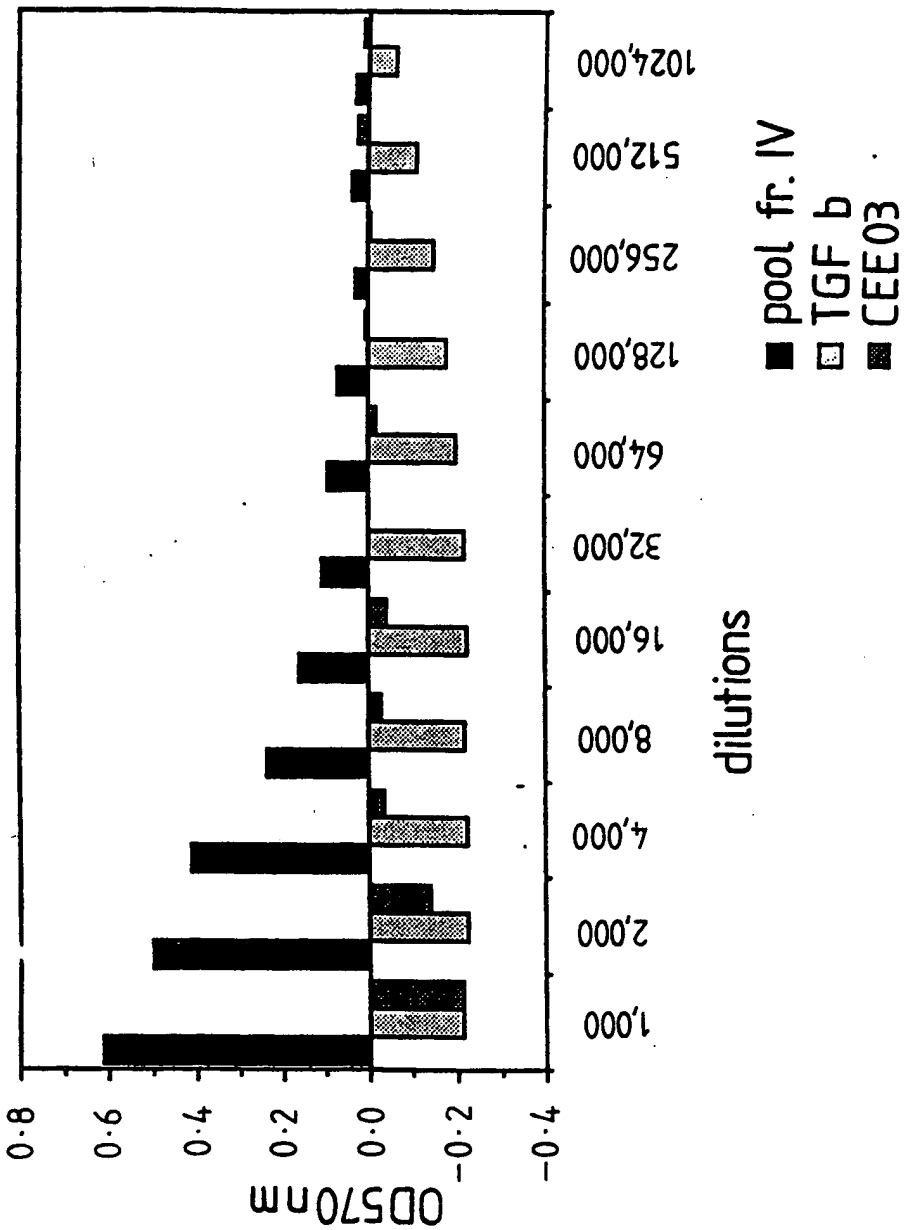

OD570nm
0·8
0·6
0·4
0·2
0·0
−0·2
−0·4
dilutions
1,000
2,000
4,000
8,000
16,000
32,000
64,000
128,000
256,000
512,000
1024,000
pool fr. IV
TGF b
CEE03

FIG 7b

Absorbance
−0·1169
−0·0387
0·0395
0·1177
0·1958
0·2740
0·35
4·04
7·73
11·41
15·10
18·79
22·47
26·16
29·85
33·53
37·22
40·91
44·59
48·28
51·97
55·65
59·34
63·02
0·1169
0·0387
0·0395

FIG 8 I

0.0029
0.0344
0.0717
0.1091
0.1464
0.1837
63.03
59.35
55.66
51.97
48.28
44.60
40.91
37.22
33.54
29.65
26.16
22.48
18.79
15.10
11.41
7.73
4.04
0.35
Absorbance

FIG 8 II

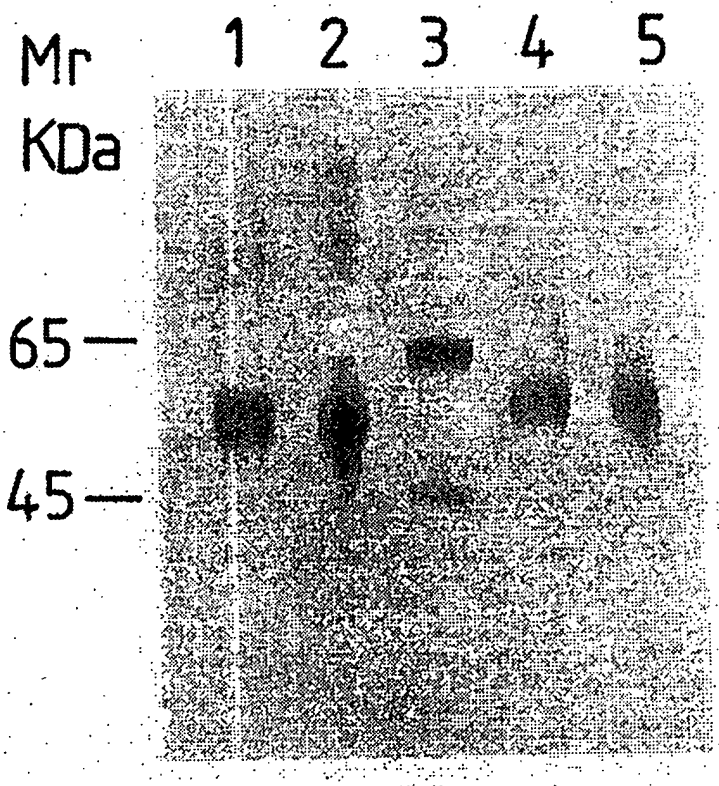
Absorbance
-0·1414
-0·0805
-0·0196
0·0412
0·1021
0·1630
0·15
1·67
3·19
4·70
6·22
7·74
9·26
10·77
12·29
13·81
15·33
16·85
18·36
19·88
21·40
22·92
24·43
25·95

FIG 8 III

0·0050
0·0225
0·0400
0·0574
0·0749
0·0923
67·88
63·91
59·94
55·97
52·00
48·03
44·06
40·09
36·12
32·15
28·18
24·20
20·23
16·26
12·29
8·32
4·35
0·38
Absorbance

FIG 9

FIG 10



A. **CLASSIFICATION OF SUBJECT MATTER**

Int. Cl.[5] C12N 5/06, 5/08; C07K 15/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. **FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

C12N 5/06, 5/08

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

AU C12N 5/06, 5/08
IPC

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)

STN: -PVAGY-
WPAT (Embr:(s)stem(w)cell#; totipotent; fibroblast; ES;
CASM (Avian(w)factor#,EMBRYO:()STEM()CELL()FACTOR#; ES()CELL()FACTOR;
CHICKEN()EMBRYONIC()FIBROBLAST#; CEF

C. **DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	WO 90/03439 (Amgen Inc.) 5 April 1990 (05.04.90)	
A	WO 91/13985 (Heath J.K.; Smith, A.G.; and Rathgen, P.D.) 19 September 1991 (19.09.91)	
A	WO 90/03432 (Animal Technology Cambridge Ltd; Institute of Animal Physiology and Genetics Research) 5 April 1990 (05.04.90)	

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 November 1993 (03.11.93)	15 NOV 1993 (15.11.93)

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